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### Authors

Chen, Chiung-Kuang  
Doyle, Patricia S  
Yermalitskaya, Liudmila V  
et al.

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# Trypanosoma cruzi CYP51 Inhibitor Derived from a Mycobacterium tuberculosis Screen Hit

Chiung-Kuang Chen<sup>1</sup>, Patricia S. Doyle<sup>2</sup>, Liudmila V. Yermalitskaya<sup>3</sup>, Zachary B. Mackey<sup>2</sup>, Kenny K. H. Ang<sup>2</sup>, James H. McKerrow<sup>2</sup>, Larissa M. Podust<sup>1\*</sup>

**1** Department of Pharmaceutical Chemistry, University of California, San Francisco, California, United States of America, **2** Sandler Center for Basic Research in Parasitic Diseases, University of California, San Francisco, California, United States of America, **3** Department of Pharmacology, Vanderbilt University, Nashville, Tennessee, United States of America

## Abstract

**Background:** The two front-line drugs for chronic *Trypanosoma cruzi* infections are limited by adverse side-effects and declining efficacy. One potential new target for Chagas' disease chemotherapy is sterol 14 $\alpha$ -demethylase (CYP51), a cytochrome P450 enzyme involved in biosynthesis of membrane sterols.

**Methodology/Principal Finding:** In a screening effort targeting *Mycobacterium tuberculosis* CYP51 (CYP51<sub>Mt</sub>), we previously identified the *N*-[4-pyridyl]-formamide moiety as a building block capable of delivering a variety of chemotypes into the CYP51 active site. In that work, the binding modes of several second generation compounds carrying this scaffold were determined by high-resolution co-crystal structures with CYP51<sub>Mt</sub>. Subsequent assays against the CYP51 orthologue in *T. cruzi*, CYP51<sub>Tc</sub>, demonstrated that two of the compounds tested in the earlier effort bound tightly to this enzyme. Both were tested *in vitro* for inhibitory effects against *T. cruzi* and the related protozoan parasite *Trypanosoma brucei*, the causative agent of African sleeping sickness. One of the compounds had potent, selective anti-*T. cruzi* activity in infected mouse macrophages. Cure of treated host cells was confirmed by prolonged incubation in the absence of the inhibiting compound. Discrimination between *T. cruzi* and *T. brucei* CYP51 by the inhibitor was largely based on the variability (phenylalanine versus isoleucine) of a single residue at a critical position in the active site.

**Conclusions/Significance:** CYP51<sub>Mt</sub>-based crystal structure analysis revealed that the functional groups of the two tightly bound compounds are likely to occupy different spaces in the CYP51 active site, suggesting the possibility of combining the beneficial features of both inhibitors in a third generation of compounds to achieve more potent and selective inhibition of CYP51<sub>Tc</sub>.

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\* E-mail: larissa.podust@ucsf.edu

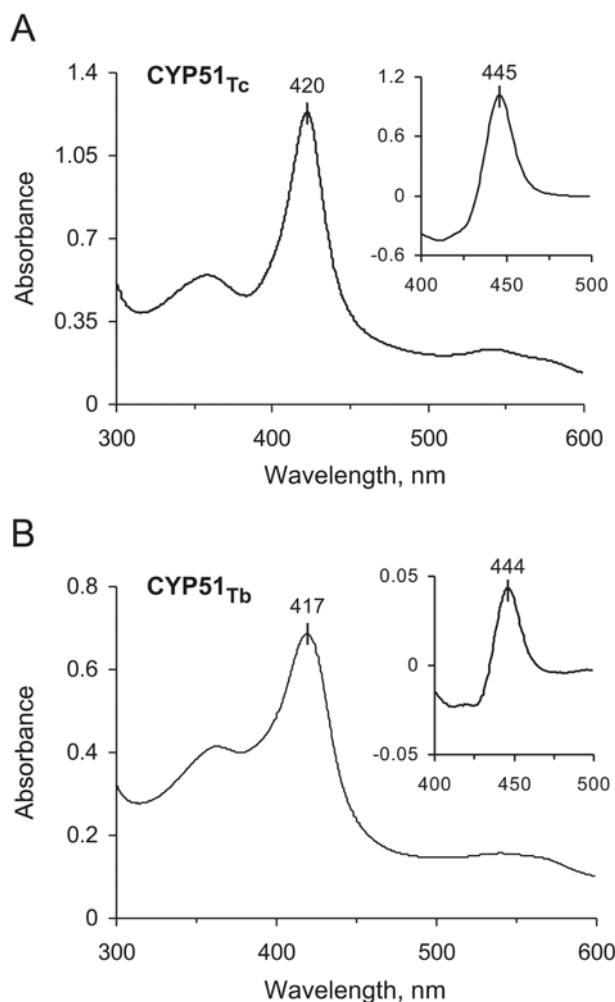
## Introduction

The drug development pipeline targeting diseases caused by trypanosome parasites is sparse [1]. Despite significant advances in its control over the last 15 years [2], Chagas' disease, caused by the parasitic protozoan *Trypanosoma cruzi* [3], remains a major public health concern in Latin America, with an estimated total of 8 million people infected [4]. Nifurtimox and benznidazole, the two principal drugs for treatment of Chagas' disease, were launched in 1967 and 1972 respectively, and suffer from the twin liabilities of serious side-effects and reduced efficacy in chronic *T. cruzi* infections [2]. A potential new target for Chagas' disease chemotherapy is sterol 14 $\alpha$ -demethylase (CYP51) [5], a cytochrome P450 heme thiolate-containing enzyme which is involved in biosynthesis of membrane sterols in all biological kingdoms from bacteria to animals [6]. *T. cruzi* sterols are similar in composition to those in fungi, with ergosterol and ergosterol-like sterols the major membrane components [7]. Clinically employed

antifungal azoles [8,9] inhibit ergosterol biosynthesis in fungi and are partially effective against *Leishmania* and *Trypanosoma* parasites [10–12]. Azoles block CYP51 activity, resulting in decline of the normal complement of endogenous sterols and accumulation of various 14 $\alpha$ -methyl sterols with cytostatic or cytotoxic consequences [11]. Aside from the compounds optimized for antifungal therapy, other CYP51 inhibitors with strong anti-*T. cruzi* activity have also been reported [13–15].

Mammalian CYP51 shares relatively modest overall sequence identity – below 30% – with its fungal and protozoan counterparts, but within the active site the amino acid residues are far more conserved. Based upon crystal structures of CYP51 of *M. tuberculosis* (CYP51<sub>Mt</sub>) [16–20], three of the thirteen active site residues, Y76, F83, and H259 (numbering according to CYP51<sub>Mt</sub>), are invariant throughout the *cyp51* gene family. Two residues, F78 and F255, are specific to the methylation status of the C-4 atom in the sterol nucleus [18,21], and amino acid identities of seven other





**Figure 1. Spectral characteristics of CYP51<sub>Tc</sub> (A) and CYP51<sub>Tb</sub> (B).** Main panel shows the absolute protein spectrum, while insert shows CO-bound reduced difference spectrum.  
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elements was inoculated with 10 ml of overnight culture and growth continued at 37°C and 250 rpm agitation until OD<sub>600</sub> reached 0.3. At that point expression of chaperones was induced with 0.2% arabinose. Growth continued at 27°C and 180 rpm until OD reached 0.6. Then CYP51<sub>Tb</sub> expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, final concentration 0.3 mM), and δ-aminolevulinic acid (1 mM). Following induction, temperature was decreased to 15°C. After 48 hours the cells were harvested and lysed by sonication. Purification was conducted similarly to as described above for CYP51<sub>Tc</sub>, with the qualification that S-Sepharose was used in the flow-through regime, while the protein was bound to and eluted from the Q-Sepharose column. Spectral characteristics of CYP51<sub>Tb</sub> are shown in Figure 1B.

### Crystallization, Data Collection, and Determination of Crystal Structure

Five compounds (Fig. 2), purchased from ChemDiv (San Diego, California) were used for co-crystallization with the CYP51<sub>Mt</sub> C37L/C442A double mutant. Compared to the wild type, this construct has superior propensity for crystallization. Compound numbering is according to the order in which they were received

in our laboratory, with number **7** being the first used in the current work. Ligands were dissolved in Me<sub>2</sub>SO at ≤100 mM stock concentration, and brought to final concentrations ranging from 1 to 5 mM in the crystallization mix, depending on ligand solubility. Protein concentration was 0.2 mM. A narrow crystallization screening grid (15–30% PEG 4000, 2–12% isopropanol, 0.1 M HEPES, pH 7.5), previously devised to obtain CYP51<sub>Mt</sub> crystals [16,18,19] was utilized for co-crystallization of complexes by the vapor diffusion hanging drop method. Four co-crystal forms were obtained, all diffracted to resolutions between 1.56 to 1.60 Å. Diffraction data were collected at 100–110 K at the Southeast Regional Collaborative Access Team (SER-CAT) 22ID beamline, Advanced Photon Source, Argonne National Laboratory using SER-CAT mail-in data collection program (Table 1). The images were integrated and the intensities merged with the HKL2000 software suite [25]. The structures were determined by molecular replacement using coordinates of estriol-bound CYP51<sub>Mt</sub> (Protein Data Bank ID 1X8V) as a search model. The final atomic models were obtained after a few iterations of refinement using REFMAC5 [26] and model-building using the COOT graphics modeling program [27]. The quality of the structures was assessed by the program PROCHECK [28]. One residue, A46, was found in the generously allowed region of the Ramachandran plot in all structures where, together with the adjacent G47, it enables a sharp turn between two β strands.

### Spectroscopic Binding Assays

Spectroscopic binding assays were performed at room temperature in 1-ml quartz cuvette containing 1 μM or 2 μM CYP51 in 50 mM Tris-HCl, pH 7.5, and 10% glycerol using a Cary UV-visible scanning spectrophotometer (Varian). Concentration of CYP51 was determined at 450 nm from the difference spectra between the carbon monoxide-bound ferrous and water-bound ferric forms, with an extinction coefficient of 91,000 M<sup>-1</sup> cm<sup>-1</sup> [29]. In the first round, compounds dissolved in Me<sub>2</sub>SO at 10 mM concentration were added to the 2 μM protein solution in 0.5 μl aliquots, resulting in concentration increases from 5 μM to 50 μM in 5 μM increments. The same amounts of Me<sub>2</sub>SO alone were added to the protein in the reference cuvette, followed by recording the difference spectra. In the second round, compounds with high affinities were diluted to 100 μM by Me<sub>2</sub>SO and titrated into 1 μM protein solution in 1 μl aliquots to increase compound concentration from 0.1 μM to 2 μM in 0.1 μM increments. To determine the *K<sub>D</sub>*, we used the GraphPad PRISM software (Graphpad Software Inc.) to fit titration data to either rectangular or quadratic hyperbolas to correct for the bound ligand fraction, according to the functions  $\Delta A = (A_{\max}(S/K_D + S))$  or  $\Delta A = (A_{\max}/2[E])((K_D + [E] + [L]) - ((K_D + [E] + [L])^2 - 4[E][L])^{1/2})$ , respectively, where E is total enzyme and L total ligand concentration, *A<sub>max</sub>* the maximal absorption shift at saturation, and *K<sub>D</sub>* the apparent dissociation constant for the enzyme-ligand complex.

### *T. cruzi* Assay

Irradiated (1000 rads) J774 mouse macrophages were plated in 12-well tissue culture plates 24 h prior to infection with 10<sup>5</sup> *T. cruzi* Y strain trypomastigotes for 2 h at 37°C. Cultures were maintained in RPMI-1640 medium with 5% heat-inactivated fetal calf serum and 5% CO<sub>2</sub> with the addition of 10 μM compound **8** or **10**. Untreated controls, controls treated with the inhibitor K11777 (10 μM) [30,31], and uninfected macrophage controls were also included. All cultures were in triplicate and medium was replaced every 48 h. Treatment with CYP51 inhibitors continued for up to 27 days. Subsequently, treated cultures were maintained without inhibitor for an additional 13–

| Compound Structure | ID ChemDiv #                   | K <sub>D</sub> , $\mu$ M |                          |                                   |                        |
|--------------------|--------------------------------|--------------------------|--------------------------|-----------------------------------|------------------------|
|                    |                                | CYP51 <sub>Mt</sub>      | CYP51 <sub>Mt-F78L</sub> | CYP51 <sub>Tc</sub>               | CYP51 <sub>Tb</sub>    |
|                    | EBPA<br>3937-0152 <sup>1</sup> | 5.2 $\pm$ 0.2            | 21.5 $\pm$ 1.0           | 0.48 $\pm$ 0.08                   | Compound not available |
|                    | Fluconazole                    | 26.0 $\pm$ 1.4           | 69.9 $\pm$ 5.3           | 0.15 $\pm$ 0.03                   | 0.034 $\pm$ 0.014      |
|                    | Compound 7<br>4130-5342        | No binding               | No binding               | 20.7 $\pm$ 0.8                    | >100                   |
|                    | Compound 8<br>4596-0233        | 58.3 $\pm$ 4.6           | 14.6 $\pm$ 1.2           | <b>0.16<math>\pm</math>0.03</b>   | <b>No binding</b>      |
|                    | Compound 9<br>C155-0065        | 35.4 $\pm$ 1.6           | 41.0 $\pm$ 1.8           | 6.9 $\pm$ 0.6                     | 6.5 $\pm$ 0.6          |
|                    | Compound 10<br>C155-0123       | 18.4 $\pm$ 2.5           | 6.3 $\pm$ 0.8            | <b>0.042<math>\pm</math>0.008</b> | <b>No binding</b>      |
|                    | Compound 11<br>C155-0337       | >100                     | >100                     | 9.2 $\pm$ 1.6                     | 61.5 $\pm$ 6.2         |

**Figure 2. Chemical structures and binding affinities of compounds.** <sup>1</sup>Compounds are identified by the numbers in the ChemDiv, Inc., product library catalog.  
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15 days to confirm inhibitor effectiveness and cure of infected cells. Cultures were monitored daily by contrast phase microscopy to determine presence of *T. cruzi* infected cells and free infectious trypomastigotes (Table 2).

### IC<sub>50</sub>

To determine IC<sub>50</sub>, mouse J774 macrophages were irradiated (1000 rads) to deter growth and plated onto 12-well tissue culture plates. Cells were infected with 10<sup>5</sup> tissue culture trypomastigotes of the Y strain of *T. cruzi* for 2 h at 37°C, as described above. Next, medium was replaced with the addition of compound **10** at 0, 1 nM, 10 nM, 100 nM, 500 nM, 1  $\mu$ M, 5  $\mu$ M, and 10  $\mu$ M; these cultures were incubated for 52 h at 37°C. Controls with 10  $\mu$ M K11777 and 10  $\mu$ M compound **8** were also included. All treatments were performed in triplicate to ensure statistical validity. Cultures were then fixed in 4% paraformaldehyde in PBS for 2 h at room temperature and stained with DAPI (10 nM) in PBS. One hundred cells and their intracellular parasites were quantified as previously described to estimate the mean number of parasites/cell [32]. Mean P/cell data were plotted against compound concentration to estimate the IC<sub>50</sub>.

### Mammalian Cell Toxicity Assay

Toxicity was evaluated in bovine muscle cells (BESM), mouse J774 macrophages, and human Huh7 hepatocytes against compound **10** at 10  $\mu$ M, 50  $\mu$ M and 100  $\mu$ M concentrations. After 48 h in culture at 37°C, cells were stained with 10% Tripan

Blue and the number of live *versus* dead cells was quantified (Table 3).

### *T. brucei* Assay

Trypanosomes were grown in complete HMI-9 medium containing 10% FBS, 10% Serum Plus medium (Sigma Inc. St. Louis Mo. USA) and 1 $\times$  penicillin/streptomycin. Trypanosomes were diluted to 1.0 $\times$ 10<sup>5</sup>/ml in complete HMI-9 medium. Diluted trypanosomes were aliquoted in Greiner sterile 96-well flat white opaque culture plates using a WellMate cell dispenser (Matrix Tech., Hudson, NH, USA). Compounds **8** and **10** were serially diluted in Me<sub>2</sub>SO. Trypanosomes were incubated with the compounds for 48 h at 37°C with 5% CO<sub>2</sub> before monitoring viability. Trypanosomes were then lysed in the wells by adding 50  $\mu$ l of CellTiter-Glo™ (Promega Inc., Madison, WI, USA). Lysed trypanosomes were placed on an orbital shaker at room temperature for 2 min. The resulting ATP-bioluminescence of the trypanosomes in the 96-well plates was measured at room temperature using an Analyst HT plate reader (Molecular Devices, Sunnyvale, CA, USA).

## Results

### Crystal Structures of CYP51<sub>Mt</sub>-Inhibitor Complexes

Co-crystals were obtained for compounds **8**, **9** and **11**. Compound **10** failed to generate any crystals with CYP51<sub>Mt</sub>. Compound **7** was not found in the CYP51<sub>Mt</sub> active site in the



**Table 1.** Crystallographic data and statistics.

| Compound #   | Compound 8                                    | Compound 9                                    | Compound 11                                   |
|--|---|---|---|
| ChemDiv #  | 4596-0233                                     | C155-0065                                     | C155-0337                                     |
| PDB ID   | 2W0B  | 2W09  | 2W0A  |
| <b>Data collection</b>                                   |   |   |   |
| Wavelength, Å  | 1.0000  | 1.0000  | 1.0000  |
| Resolution, Å  | 1.56  | 1.57  | 1.60  |
| Unique reflections                                       | 63021   | 59811   | 53704   |
| Redundancy <sup>a</sup>                                  | 5.5 (4.7)                                     | 5.4 (3.4)                                     | 5.1 (3.9)                                     |
| Completeness, %  | 99.5 (99.1)                                   | 95.8 (79.4)                                   | 93.8 (77.7)                                   |
| Space group  | P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub> | P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub> | P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub> |
| Cell dimensions (a, b, c), Å                             | 46.7, 84.8, 110.3                             | 46.4, 85.1, 110.9                             | 44.7, 85.7, 110.9                             |
| Molecules in asymmetric unit                             | 1   | 1   | 1   |
| Solvent content, %                                       | 40  | 40  | 40  |
| R <sub>sym</sub> <sup>b</sup> , %                        | 5.2 (29.7)                                    | 7.7 (32.8)                                    | 7.0 (32.2)                                    |
| I/σ  | 32.1 (4.3)                                    | 45.5 (4.4)                                    | 32.5 (3.4)                                    |
| <b>Refinement</b>  |   |   |   |
| Reflections used in refinement                           | 62962   | 59743   | 53638   |
| R <sub>cryst</sub> (R <sub>free</sub> ) <sup>c</sup> , % | 16.4/20.1                                     | 18.8/22.2                                     | 18.9/23.2                                     |
| No. of atoms   | 3989  | 3753  | 3726  |
| Protein  | 3542  | 3409  | 3376  |
| Heme   | 43  | 43  | 43  |
| Substrate  | 24  | 24  | 22  |
| Water  | 380   | 277   | 285   |
| Wilson plot B-values, Å <sup>2</sup>                     | 15.2  | 22.6  | 18.7  |
| Mean B-factor, Å <sup>2</sup>                            | 14.6  | 25.5  | 20.3  |
| Protein  | 14.0  | 24.9  | 19.7  |
| Heme   | 9.6   | 22.6  | 21.0  |
| Substrate  | 14.9  | 29.0  | 21.0  |
| Water  | 22.8  | 32.6  | 27.8  |
| <b>r.m.s. deviations</b>                                 |   |   |   |
| Bond length, Å   | 0.010   | 0.013   | 0.013   |
| Bond angles, °   | 1.3   | 1.4   | 1.4   |
| Ramachandran (%) <sup>d</sup>                            | 91.9/7.9/0.3                                  | 91.7/8.0/0.3                                  | 91.5/8.2/0.3                                  |

<sup>a</sup>Numbers in parentheses correspond to the highest resolution shell.

<sup>b</sup>R<sub>sym</sub> =  $\sum |I_i - \langle I \rangle| / \sum I_i$ , where  $I_i$  is the intensity of the  $i^{\text{th}}$  observation, and  $\langle I \rangle$  is the mean intensity of reflection.

<sup>c</sup>R<sub>cryst</sub> =  $\sum |F_o| - |F_c| / \sum |F_o|$ , calculated with the working reflection set. R<sub>free</sub> is the same as R<sub>cryst</sub> but calculated with the reserved reflection set.

<sup>d</sup>Program PROCHECK [28], portions of the protein residues in most favored/additional allowed/generously allowed regions.

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crystal, which is consistent with lack of spectrally detectable binding (Fig. 2). Compounds **8** (3-[[4-methylphenyl)sulfonyl]amino]propylpyridin-4-ylcarbamate), **9** (cis-4-methyl-N-[(1S)-3-(methylsulfonyl)-1-(pyridin-4-ylcarbamoyl)propyl]cyclohexanecarboxamide), and **11** (N-[(1S)-2-methyl-1-(pyridin-4-ylcarbamoyl)propyl]cyclohexanecarboxamide), were observed bound in the CYP51<sub>Mt</sub> active site as predicted, through the coordination of the heme iron via a lone pair of aromatic nitrogen electrons of the *N*-[4-pyridyl]-formamide moiety (highlighted in *gray* in Fig. 2) and interactions with the invariant residues Y76 and H259 (Fig. 3). Functional groups other than the *N*-[4-pyridyl]-formamide moiety

**Table 2.** *T. cruzi* infection *in vitro*.

| Treatment                  | Host cell survival | <i>T. cruzi</i> development |
|----------------------------|--------------------|-----------------------------|
| Untreated control          | 5 days             | 5 days                      |
| Compound <b>8</b> (10 μM)  | 5 days             | 5 days                      |
| Compound <b>10</b> (10 μM) | 40 days            | No                          |
| K11777 control             | 40 days            | No                          |

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in compounds **9** and **11** either were accommodated in the species-specific cavity or else protruded through the opening of the active site toward bulk solvent. H259 hydrogen-bonded to the carbonyl oxygen in both compounds, while interactions with Y76 were mediated by two similarly positioned water molecules (Figs. 3A and 3B). The residual F<sub>o</sub>-F<sub>c</sub> electron density map suggested two alternative conformations for compounds **11** and **9**, designated by *pink* and *cyan* respectively in Figures 4A and 4B. In the CYP51<sub>Mt</sub>-compound **11** complex, the cyclohexane ring protruded toward the bulk solvent (Fig. 3A), barely interacting with the protein in two alternative conformations (Fig. 4A). Together with the limited interactions of the isopropyl moiety, this lack of contact explains the low binding affinity of **11**. In the CYP51<sub>Mt</sub>-compound **9** complex, the methylcyclohexane moiety protruded toward bulk solvent, while the methylsulfonyl group loosely bound in the species-specific cavity (Fig. 3B) in two alternative conformations (Fig. 4B). The side chain of M433 also adopted two alternative conformations. In both complexes, a portion of the BC-region was disordered and missing from the electron density map. Although racemic mixtures were used for co-crystallization, only one enantiomer of each compound was found in the active site.

A different binding mode was revealed for compound **8**. Its flexible backbone allowed it to fold head-to-tail over the heme plane to bring the methylphenylsulfonamide group into intramolecular stacking interactions with the pyridinyl moiety and also with the heme macrocycle (Fig. 3C, 4C). Folding minimized the nonpolar surface of compound **8** by exposing the sulfonamide group to interactions with Q72, K97, and the heme propionate side chain. The hydrophobic side chain of K97 aligned along the methylphenyl moiety. A similar folding of the benzothiadiazolsulfonamide group has been observed in previous work for 2-[(2,1,3-benzothiadiazol-4-sulfonamide)-2-phenyl-N-pyridin-4-acetamide (BSPPA) [19]. Mutually stabilizing protein-ligand interactions involving the BC-loop residues including F78 result in increased binding affinity of the CYP51<sub>Mt</sub>-compound **8** complex and in unambiguous electron density both for compound **8** (Fig. 3C) and for the entire BC-region. In the CYP51<sub>Mt</sub>-compound **8** complex, H259 directly H-bonded to the amide nitrogen of compound **8**, whereas Y76 interacted hydrophobically with the compound's flexible backbone (Fig. 3C).

### Binding Affinities of CYP51 Ligands

Binding affinities of all five compounds were examined against both wild type and a 'humanized' F78L mutant form of CYP51<sub>Mt</sub>, CYP51<sub>Tc</sub>, and CYP51<sub>Tb</sub> using spectroscopic assays (Fig. 5). These assays utilize the property of P450 enzymes to shift the ferric heme iron Soret band following replacement of a weak ligand, the water molecule, with a stronger one, the nitrogen-containing aromatic pyridinyl group (Fig. 5A). All compounds had markedly reduced or no binding affinity toward CYP51<sub>Mt</sub>, compared to the parental EPBA (Fig. 2). No binding was observed for compound **7**, while

**Table 3.** Toxicity in mammalian cells.

| Treatment                 | Mean Live Cells $\pm$ SD |                     |                     |
|---------------------------|--------------------------|---------------------|---------------------|
|                           | BESM (bovine)            | Macrophages (mouse) | Hepatocytes (human) |
| Untreated control         | 89 $\pm$ 5               | 97.5 $\pm$ 0.8      | 92.5 $\pm$ 0.8      |
| Compound 10 (10 $\mu$ M)  | 88.5 $\pm$ 0.5           | 96 $\pm$ 0.1        | 94 $\pm$ 1          |
| Compound 10 (50 $\mu$ M)  | 62.5 $\pm$ 0.8           | 91 $\pm$ 0.1        | 83 $\pm$ 3          |
| Compound 10 (100 $\mu$ M) | 10 $\pm$ 1               | 15 $\pm$ 0.5        | 53 $\pm$ 1          |

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the  $K_D$  for compound **11** exceeded 100  $\mu$ M, indicating weak binding.

However, the binding affinity of all compounds examined, including compound **7**, was significantly higher to CYP51<sub>Tc</sub> than to CYP51<sub>Mt</sub>. Remarkably, the binding affinities of compounds **8** and **10** to CYP51<sub>Tc</sub> were 300- and at least 500-fold respectively higher, equaling or exceeding that of the antifungal CYP51 inhibitor fluconazole, which was used as a reference (Fig. 5B, C). A  $K_D$  of at least 40 nM was estimated for compound **10** by spectral assays, with the binding curve reaching a plateau at about a 1:1 protein to ligand ratio. This value strongly suggests that the  $K_D$  must be notably higher, although further dilution of protein in an attempt to obtain a more accurate value significantly decreased the quality of the spectra and this effort was thus abandoned. The IC<sub>50</sub> of  $\sim$ 1 nM for *T. cruzi* intracellular growth inhibition, determined for compound **10** as described below, may better reflect true  $K_D$  value. Compounds **8** and **10**, which had highest binding affinity to CYP51<sub>Tc</sub>, were spectrally silent toward CYP51<sub>Tb</sub>, indicating no binding in the active site (Fig. 2). As expected, CYP51<sub>Tb</sub> had nanomolar affinity for fluconazole (Fig. 5D), but again, the plateau was reached at a 1:1 protein to inhibitor ratio, so the binding constant could not be determined more accurately. Compound **9** bound both CYP51<sub>Tc</sub> and CYP51<sub>Tb</sub> with the same affinity.

The  $K_D$  values for compounds **8** and **10** slightly decreased for the F78L CYP51<sub>Mt</sub> mutant compared to the wild type, while the  $K_D$  values for the other compounds increased (Fig. 2).

### Inhibitory Effects against *T. cruzi*

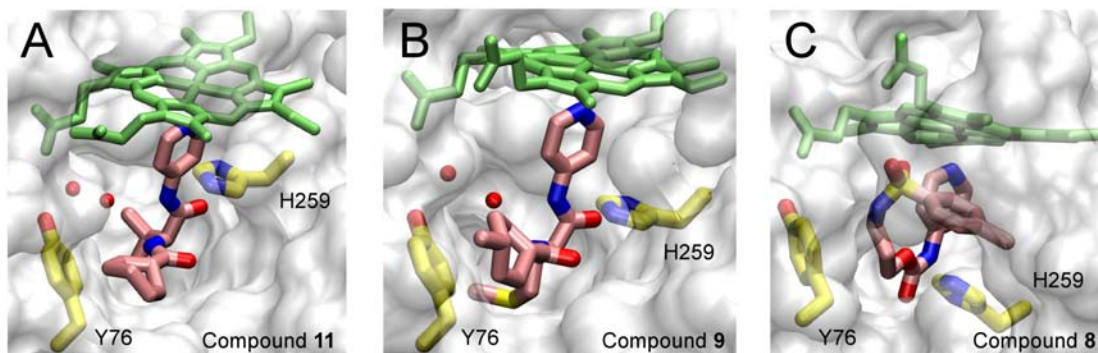
With submicromolar affinities toward CYP51<sub>Tc</sub> of 160 nM and  $<$ 40 nM respectively, compounds **8** and **10** were examined *in vitro*

for inhibitory effects against both *T. cruzi* and *T. brucei*. In a mouse macrophage assay, *T. cruzi* completed its intracellular development in 5 days in untreated controls, resulting in death of host macrophages and abundant trypomastigotes in culture supernatant (Table 2). As anticipated, the control compound K11777 [30] cured *T. cruzi* infection. No parasites survived a treatment regime of 27 days with compound **10**. Cure of host cells was confirmed by incubation of the cultures for an additional 15 days in the absence of inhibitor. In contrast, and similarly to untreated controls, *T. cruzi* completed its development in 5 days in cultures treated with compound **8**.

An IC<sub>50</sub> of  $\sim$ 1 nM concentration for compound **10** (Fig. 6) was estimated for *T. cruzi* intracellular amastigotes. *T. cruzi* developed well intracellularly in untreated macrophages with a final mean number of 3.57 $\pm$ 0.5 P/cell (0% inhibition). As determined previously, 10  $\mu$ M compound **10** was deleterious for *T. cruzi*, with a mean of 0.25 $\pm$ 0.01 P/cell (100% growth inhibition). Ten  $\mu$ M of control compound K11777 was also parasitocidal for *T. cruzi* with a mean of 0.25 $\pm$ 0.01 P/cell (IC<sub>100</sub>) [30], while compound **8** was not parasitocidal at this concentration with a mean of 1.22 $\pm$ 0.1 P/cell (data not shown).

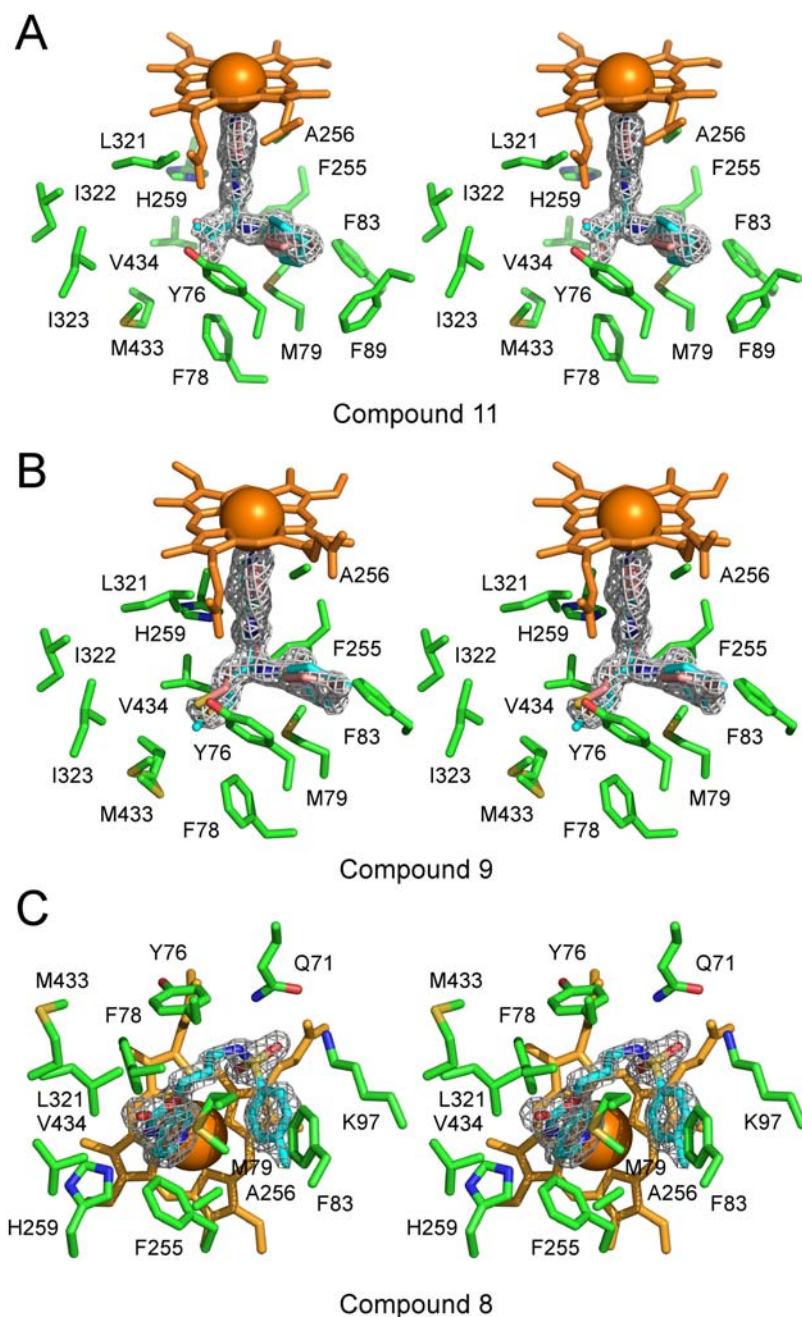
Toxicity for mammalian cells was addressed by treating the three different cell types with increasing concentrations of compound **10** (Table 3). No toxicity was observed at 10  $\mu$ M compound **10**, while 50  $\mu$ M was mildly toxic for muscle cells. One hundred  $\mu$ M compound **10** was toxic for all mammalian cells tested, especially muscle cells.

Consistent with the spectral binding assays, neither compound **8** nor **10** had any inhibitory effects against cultured *T. brucei* even at the highest tested concentration of 10  $\mu$ M.



**Figure 3. Overall view of compound binding in the active site.** Compounds **11** (A), **9** (B), and **8** (C) (highlighted in pink) bound in the active site of CYP51<sub>Mt</sub> are shown looking in from the active site opening. For clarity only one conformation of each compound is shown. Protein is represented by the semitransparent accessible surface (gray). The ordered BC-loop obstructs the view in the CYP51<sub>Mt</sub>-**8** complex in (C). The invariable elements of the CYP51 active site, Y76, H259 (yellow), and heme (green), are in a stick mode. Water molecules are shown as red spheres. Oxygen atoms are red, nitrogen blue, sulfur yellow. Images were generated using the VMD program [35].

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**Figure 4. Stereo view of compounds in the active site.** Compounds **11** (A), **9** (B), and **8** (C) are shown surrounded by the CYP51 active site residues. The fragments of the electron density 2Fo-Fc map (gray mesh) are cut at 1.2  $\sigma$ . Different conformers in (A) and (B) are highlighted in pink and cyan. Images were generated using PYMOL [36].  
doi:10.1371/journal.pntd.0000372.g004

#### Protein Data Bank Accession Numbers

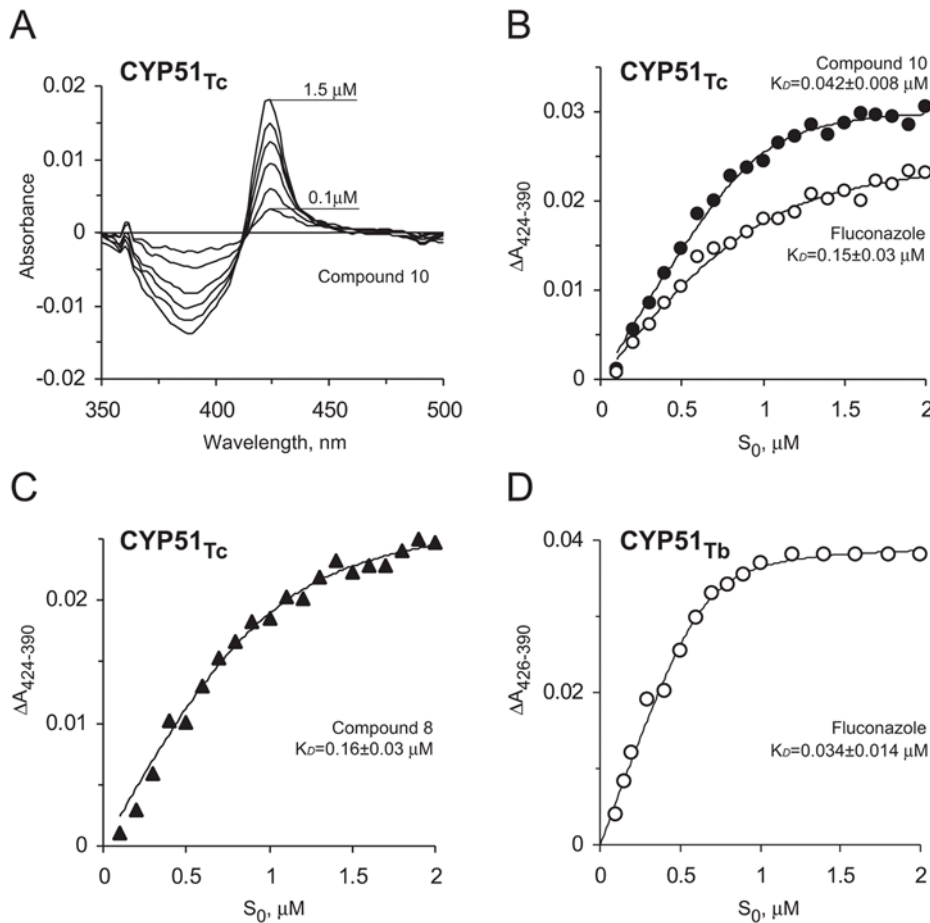
The atomic coordinates and structure factors determined in this study (Protein Data Bank IDs 2W09, 2W0A, and 2W0B) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

#### Discussion

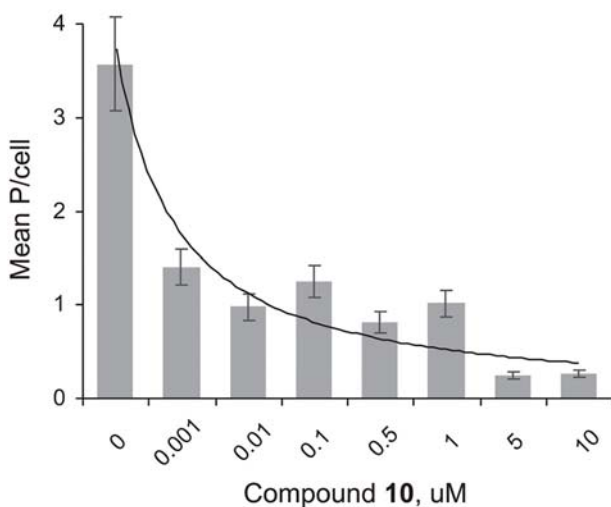
We explored sterol 14 $\alpha$ -demethylase (CYP51) as a potential target for trypanosomiasis chemotherapy by probing CYP51<sub>MT</sub>,

CYP51<sub>TC</sub>, and CYP51<sub>TC</sub> with second generation compounds that contain a universal building block, the *N*-[4-pyridyl]-formamide moiety, which is capable of delivering small molecule compounds to the CYP51 active site. The affinities of the *N*-[4-pyridyl]-formamide-derivative compounds that we tested against CYP51<sub>MT</sub> were lower than that of EPBA (Fig. 2), from which the formamide building block was derived. Affinities of all compounds examined were much higher toward CYP51<sub>TC</sub> than to CYP51<sub>MT</sub>. Strikingly large increases in binding affinities – 300 and 500 fold – were observed for compounds **8** and **10**. Although compound **10** did not produce crystals with CYP51<sub>MT</sub>, based on the binding modes





**Figure 5. Spectroscopic binding of compounds.** (A) Type II spectral responses of CYP51<sub>Tc</sub> to increasing concentrations of compound 10. The concentration dependence of compound 10, fluconazole (B), and compound 8 (C) binding were deduced from the difference absorption changes obtained from the titration of CYP51<sub>Tc</sub> with increasing concentrations of the inhibitor. The concentration dependence of fluconazole (D) was deduced from the difference absorption changes obtained from the titration of CYP51<sub>Tb</sub>. doi:10.1371/journal.pntd.0000372.g005



**Figure 6. Inhibition of *T. cruzi* intracellular amastigotes by compound 10.** *T. cruzi* intracellular multiplication was evaluated at 52 hr of incubation at several concentrations of the inhibitor by determining the number of parasites/cell. Intracellular parasites were counted per one hundred cells to estimate a mean number of parasites per cell. Approximation of concentration dependence of mean P/cell  $\pm$  SD data with a smooth curve highlights the 50% drop in parasite count at  $\sim 1$  nM compound 10. SD did not exceed 14% of the mean. doi:10.1371/journal.pntd.0000372.g006

of compounds **9** and **11**, we reason that the methylcyclohexanecarboxamide moiety of compound **10** protrudes toward the BC-loop, suggesting that the indole ring binds in the species-specific cavity, including the space occupied in CYP51<sub>Mt</sub> by the F78 aromatic ring, which is absent from CYP51<sub>Tc</sub> but present in CYP51<sub>Tb</sub> and CYP51<sub>Mt</sub>. Consistent with this hypothesis, compound **10** selectively bound CYP51<sub>Tc</sub>, inhibited *T. cruzi* growth with the IC<sub>50</sub> value close to the K<sub>D</sub> estimated in the spectral binding assays, and cured mouse macrophages infected with *T. cruzi* Y strain at 10  $\mu$ M concentration without harming them.

In contrast, compound **10** failed to bind CYP51<sub>Tb</sub> despite the identity of 12 of the 13 active site substrate binding residues, and 83% overall sequence identity between *T. cruzi* and *T. brucei* CYP51 orthologues. This result is a striking indication of the sensitivity of CYP51 to alterations of the topography of its active site at position 78. The difference in position 78 is of functional importance, because phenylalanine at this site is strictly specific to protozoa and plant species metabolizing 4 $\alpha$ -methylated sterols [18]. Interestingly, *T. cruzi* is the only protozoan where the corresponding position (position 105 according to *T. cruzi* numbering) is occupied by isoleucine. Consistent with this observation, CYP51<sub>Tc</sub> is catalytically more closely related to its fungal and animal orthologues, preferentially converting 4 $\alpha$ , $\beta$ -dimethylated sterol substrates [21], whereas *T. brucei* CYP51 is strictly specific to 4 $\alpha$ -methylated obtusifolius and norlanosterol [33]. The proteobacterium *Methylococcus capsulatus*, known to synthesize sterols from squalene [34], is the only other known organism having isoleucine in the CYP51 position corresponding to F78. Not surprising, compound **10** was inactive against *T. brucei* in inhibitory assays *in vitro*.

In humans and animals metabolizing 4 $\alpha$ , $\beta$ -dimethylated 24,25-dihydrolanosterol, position 78 is always occupied by leucine. Therefore, the F78L substitution in the CYP51<sub>Mt</sub> binding site was examined and found to slightly increase binding affinities toward compounds **8** and **10**, as opposed to the rest of the compounds whose binding affinities decreased (Fig. 2). Although a single amino acid substitution does not by any means convert bacterial

protein into its mammalian counterpart, this finding is consistent with lack of toxicity in mammalian cells at inhibitory concentrations, and supports the possibility of rational design of highly selective anti-protozoan CYP51 inhibitors. The latter is of particular pharmacological importance as far as host-pathogen cross-reactivity is concerned, since CYP51 is present in human host.

The increased binding affinities toward CYP51<sub>Tc</sub> of all the compounds we tested may indicate more extensive involvement of the BC-loop and C helix in protein-inhibitor interactions in CYP51<sub>Tc</sub> than in CYP51<sub>Mt</sub>. Assuming that compound **8** binds CYP51<sub>Tc</sub> in a similarly compact donut-like shape that fills the space adjacent to the porphyrin ring, its 300-fold increase in binding affinity could be achieved solely by stabilization of the BC-region of CYP51<sub>Tc</sub> without engaging the species-specific cavity. This possibility opens the door to a rational design effort in which the beneficial features of both compounds **8** and **10** would be combined to yield third generation compounds that would more potently and selectively inhibit CYP51<sub>Tc</sub>. Toward this end compound **10** is currently being evaluated in animal models of Chagas' disease.

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## Author Contributions

Conceived and designed the experiments: PSD JHM LMP. Performed the experiments: CKC PSD LVY ZBM KKHA LMP. Analyzed the data: PSD ZBM LMP. Contributed reagents/materials/analysis tools: PSD JHM LMP. Wrote the paper: LMP.

## References

- Nwaka S, Hudson A (2006) Innovative lead discovery strategies for tropical diseases. *Nat Rev Drug Discov* 5: 941–955.
- Schofield CJ, Jannin J, Salvatella R (2006) The future of Chagas disease control. *Trends Parasitol* 22: 583–588.
- Chagas C (1909) Nova trypanosomíase humana. *Gaceta Medica da Bahia* 40: 433–440.
- Organization WH (2002) Control of Chagas disease: second report of the WHO expert committee. Geneva: WHO.
- Docampo R, Schmunis GA (1997) Sterol biosynthesis inhibitors: potential chemotherapeutics against Chagas disease. *Parasitol Today* 13: 129–130.
- Aoyama Y (2005) Recent progress in the CYP51 research focusing on its unique evolutionary and functional characteristics as a diversozyme P450. *Front Biosci* 10: 1546–1557.
- Furlong ST (1989) Sterols of parasitic protozoa and helminths. *Exp Parasitol* 68: 482–485.
- Georgopapadakou NH (1998) Antifungals: mechanism of action and resistance, established and novel drugs. *Curr Opin Microbiol* 1: 547–557.
- Sheehan DJ, Hitchcock CA, Sibley CM (1999) Current and emerging azole antifungal agents. *Clin Microbiol Rev* 12: 40–79.
- Mishra J, Saxena A, Singh S (2007) Chemotherapy of leishmaniasis: past, present and future. *Curr Med Chem* 14: 1153–1169.
- Roberts CW, McLeod R, Rice DW, Ginger M, Chance ML, et al. (2003) Fatty acid and sterol metabolism: potential antimicrobial targets in apicomplexan and trypanosomatid parasitic protozoa. *Mol Biochem Parasitol* 126: 129–142.
- Maya JD, Cassels BK, Iturriaga-Vasquez P, Ferreira J, Faundez M, et al. (2007) Mode of action of natural and synthetic drugs against *Trypanosoma cruzi* and their interaction with the mammalian host. *Comp Biochem Physiol A Mol Integr Physiol* 146: 601–620.
- Urbina JA, Payares G, Molina J, Sanoja C, Liendo A, et al. (1996) Cure of short- and long-term experimental Chagas' disease using D0870. *Science* 273: 969–971.
- Buckner F, Yokoyama K, Lockman J, Aikenhead K, Ohkanda J, et al. (2003) A class of sterol 14-demethylase inhibitors as anti-*Trypanosoma cruzi* agents. *Proc Natl Acad Sci U S A* 100: 15149–15153.
- Urbina JA, Payares G, Sanoja C, Molina J, Lira R, et al. (2003) Parasitological cure of acute and chronic experimental Chagas disease using the long-acting experimental triazole TAK-187. Activity against drug-resistant *Trypanosoma cruzi* strains. *Int J Antimicrob Agents* 21: 39–48.
- Podust LM, Poulos TL, Waterman MR (2001) Crystal structure of cytochrome P450 14 $\alpha$ -sterol demethylase (CYP51) from *Mycobacterium tuberculosis* in complex with azole inhibitors. *Proc Natl Acad Sci USA* 98: 3068–3073.
- Podust LM, Stojan J, Poulos TL, Waterman MR (2001) Substrate recognition sites in 14 $\alpha$ -sterol demethylase from comparative analysis of amino acid sequences and X-ray structure of *Mycobacterium tuberculosis* CYP51. *J Inorg Biochem* 87: 227–235.
- Podust LM, Yermolitskaya LV, Lepesheva GI, Podust VN, Dalmasso EA, et al. (2004) Estriol bound and ligand-free structures of sterol 14 $\alpha$ -demethylase. *Structure* 12: 1937–1945.
- Podust LM, von Kries JP, Nasser Eddine A, Kim Y, Yermolitskaya LV, et al. (2007) Small molecule scaffolds for CYP51 inhibitors identified by high-throughput screening and defined by x-ray crystallography. *Antimicrob Agents Chemother* 51: 3915–3923.
- Nasser Eddine A, von Kries JP, Podust MV, Warrier T, Kaufmann SH, et al. (2008) X-ray structure of 4,4'-dihydroxybenzophenone mimicking sterol substrate in the active site of sterol 14 $\alpha$ -demethylase (CYP51). *J Biol Chem* 283: 15152–15159.
- Lepesheva GI, Zaitseva NG, Nes WD, Zhou W, Arase M, et al. (2006) CYP51 from *Trypanosoma cruzi*: a phyla-specific residue in the B' helix defines substrate preferences of sterol 14 $\alpha$ -demethylase. *J Biol Chem* 281: 3577–3585.
- Buckner FS, Joubert BM, Boyle SM, Eastman RT, Verlinde CL, et al. (2003) Cloning and analysis of *Trypanosoma cruzi* lanosterol 14 $\alpha$ -demethylase. *Mol Biochem Parasitol* 132: 75–81.

23. von Wachenfeldt C, Richardson TH, Cosme J, Johnson EF (1997) Microsomal P450 2C3 is expressed as a soluble dimer in *Escherichia coli* following modification of its N-terminus. *Arch Biochem Biophys* 339: 107–114.
24. Barnes HJ, Arlotto MP, Waterman MR (1991) Expression and enzymatic activity of recombinant cytochrome P450 17 alpha-hydroxylase in *Escherichia coli*. *Proc Natl Acad Sci U S A* 88: 5597–5601.
25. Otwinowski Z, Minor W (1997) Processing of x-ray diffraction data collected in oscillation mode. *Methods Enzymol* 276: 307–326.
26. Murshudov GN, Vagin AA, Dodson EJ (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr* 53: 240–255.
27. Emsley P, Cowtan K (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 60: 2126–2132.
28. Laskowski RA, MacArthur MW, Moss DS, Thornton JM (1993) PROCHECK: a program to check the stereochemical quality of protein structures. *J Appl Crystallogr* 26: 283–291.
29. Omura T, Sato R (1964) The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. *J Biol Chem* 239: 2379–2385.
30. Engel JC, Doyle PS, Hsieh I, McKerrow JH (1998) Cysteine protease inhibitors cure an experimental *Trypanosoma cruzi* infection. *J Exp Med* 188: 725–734.
31. Doyle PS, Zhou YM, Engel JC, McKerrow JH (2007) A cysteine protease inhibitor cures Chagas' disease in an immunodeficient-mouse model of infection. *Antimicrob Agents Chemother* 51: 3932–3939.
32. Doyle PS, Dvorak JA, Engel JC (1984) *Trypanosoma cruzi*: quantification and analysis of the infectivity of cloned stocks. *J Protozool* 31: 280–283.
33. Lepesheva GI, Nes WD, Zhou W, Hill GC, Waterman MR (2004) CYP51 from *Trypanosoma brucei* is obtusifoliol-specific. *Biochemistry* 43: 10789–10799.
34. Bird CW, Lynch JM, Pirt FJ, Reid WW (1971) Steroids and squalene in *Methylococcus capsulatus* grown on methane. *Nature* 230: 473–474.
35. Humphrey W, Dalke A, Schulten K (1996) VMD: visual molecular dynamics. *J Mol Graph* 14: 33–38.
36. DeLano WL (2002) The PyMOL molecular graphics system. San Carlos, CA, USA: DeLano Scientific.